REGULATION OF IMMEDIATE EARLY AND DELAYED EARLY RNA SYNTHESIS IN BACTERIOPHAGE T4-INFECTED ESCHERICHIA COLI CELLS

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To my Mother

Abbreviations used:

dCMP, dCDP, dCTP:	deoxycytidine 5′-mono-, -di- and -triphosphate, respectively.
dHMP, dHDP, dHTP:	hydroxymethyldeoxycytidine 5′-mono-, -di- and -triphosphate, respectively.
ts:	temperature sensitive.
am:	nonsense mutation, amber (UAG).

This survey is based on the following papers.

I Regulation of Early mRNA Synthesis After Bacteriophage T4 Infection of Escherichia coli. Linder, C.H., and R. Fast. 1975 J.Virol. 16: 463-469

II Evidence for a Diffusible T4 Bacteriophage Protein Governing the Initiation of Delayed Early RNA Synthesis. Linder, C.H., and O.Sköld. 1977 J.Virol. 21: 7-15

III Initiation of Delayed Early RNA Synthesis at Bacteriophage T4 Infection. Involvement of the Phage mot gene and the rho gene of the Host. Linder, C.H., and O.Sköld. 1977

IV Studies on the Regulatory Function of regA in Bacteriophage T4. Linder,C.H., and O.Sköld. 1977

In the text these papers will be referred to by their Roman numerals.

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Introduction

After bacteriophage T4 infection of the common enteric bacterium Escherichia coli, there is a sequential appearance of different viral proteins at fixed times after infection. The present investigation was aimed at elucidating some of the regulatory functions at the transcriptional level behind this pattern.

The genetic information of bacteriophage T4 is contained in one double stranded DNA molecule. T4 DNA contains 5-hydroxymethylcytosine instead of cytosine. The hydroxymethylcytosine residues are glucosylated in the DNA. By the glucosylation T4 DNA escapes the degradative action of restriction enzymes in the E.coli cell.

After adsorption to receptors on the cell surface of an Escherichia coli cell, the T4 DNA is injected into the cell. Almost immediately after infection, the host macromolecular synthesis is shut off. Different groups of T4 genes are then transcribed at different times by the host RNA polymerase. After T4 infection the host specific RNA synthesis is replaced by the synthesis of T4 specific labile mRNA and a minor fraction of T4 specific tRNA.

The so called early mRNA codes for the enzymes involved in phage DNA synthesis, which starts at about 5 min after infection at 37 C. At this time also late mRNA synthesis starts.

This type of mRNA mostly codes for phage structural proteins. During the late period of infection, progeny phages are built up in the cell by an assembly process of phage structural proteins and phage DNA. After about 30 min the infection is terminated by lysis of the cells, and the liberation of about 200 daughter phages.

The present investigation concerns the regulation of the synthesis of early mRNA. It is shown that the "turn on" of early mRNA synthesis seems to be regulated in at least three different ways:

I) immediate early mRNA synthesis, which does not seem to require phage protein synthesis for its formation.

II) delayed early mRNA synthesis, which normally requires phage protein synthesis for its formation, but the need of phage protein synthesis can be relieved by a mutation in the rho gene in the E.coli host cell.

III) delayed early mRNA synthesis, which seems to require the phage mot gene product for its formation.

The shut off of early enzyme synthesis seems to be due to alteration of the phage translational machinery, and the degradation of early mRNA late in infection.

The types of mRNA, which have been studied in this work, code for the early enzymes involved in the pathway from dCTP to dHTP;

dCTP $\xrightarrow{\text{dCTPase}}$ dCMP $\xrightarrow{\text{dCMP HMase}}$ dHMP $\xrightarrow{\text{deoxynucleotide kinase}}$ dHDP $\rightarrow$ dHTP

The last step is performed by a host enzyme.

Classification of early T4 RNA (Paper I + II)

After T4 infection the host RNA polymerase starts transcription at the so called immediate early promoters on the T4 DNA chromosome. The immediate early genes are transcribed into the immediate early class of RNA. These genes can also be transcribed in the presence of chloramphenicol as shown by hybridization experiments, by Salser et al. (15). Since immediate early mRNA can be synthesized in the presence of chloramphenicol its formation cannot require the synthesis of phage proteins.

The immediate early class of mRNA is normally followed by the delayed early class of mRNA, the synthesis of which starts at about 2 min. This class of mRNA is not synthesized in the presence of chloramphenicol (15), i.e. it can require phage protein synthesis for its formation.

Thus by the aid of chloramphenicol different types of early mRNA could be classified into the two classes, immediate early and delayed early.

The messengers coding for dCMP HMase, dCTPase and deoxynucleotide kinase were classified by an enzyme forming capacity method, where the amounts of different early messengers, synthesized during chloramphenicol treatment, were determined by their capacity to form active enzyme after addition of rifampin and removal of chloramphenicol. A messenger which was formed in spite of chloramphenicol treatment was classified as

immediate early, while early messengers which were not formed in the presence of this drug belong to the delayed early class of RNA.

Chloramphenicol was added to E.coli cells prior to T4 infection and RNA synthesis was allowed to proceed for different time periods. Rifampin was added to stop further RNA synthesis, and the chloramphenicol was removed by filtration, to allow the early RNA synthesized during the chloramphenicol treatment to express itself into enzyme activity.

It was found that dCMP HMase mRNA was synthesized during chloramphenicol treatment. It therefore belongs to the immediate early class of mRNA. The messengers coding for dCTPase and deoxynucleotide kinase could, however, not be synthesized during chloramphenicol treatment. It was concluded that they belong to the delayed early class of mRNA. These results are consistent with those of Peterson et al.(10).

Protein synthesis is needed for the formation of delayed early RNA in T4 (Paper I + II)

Many early genes of bacteriophage T4 are transcribed into polycistronic messengers, which consist of an immediate early RNA at the 5´ end and delayed early RNA at the 3´end (1). As shown above delayed early RNA synthesis is blocked in the presence of chloramphenicol. This phenomenon could be due to

I) degradation of the 3´ends of the polycistronic messengers in the presence of chloramphenicol, whereby only immediate early sequences are conserved, or

II) need of phage protein for the elongation of transcription into the delayed early genes.

Morse (8) showed that in uninfected bacteria only the sequences at the 5´ends of polycistronic messengers were conserved during chloramphenicol treatment. If protein synthesis was blocked by imposing amino acid starvation, however, the whole polycistronic messenger was conserved (9).
From this point of view we were interested to find out if delayed early RNA is synthesized during amino acid starvation.

Leucine starvation of a leucine requiring strain of E.coli was imposed prior to T4 infection, and the cells were allowed to accumulate early mRNA for 9 min. Rifampin was added and 1 min later the block of protein synthesis was relieved by addition of leucine.

The enzyme forming capacities of dCMP HMase mRNA (immediate early) and deoxynucleotide kinase mRNA (delayed early), were determined. It was found that the immediate early messenger coding for dCMP HMase is formed during leucine starvation, but not the delayed early messenger coding for deoxynucleotide kinase.

An E.coli strain carrying a suA mutation is said to conserve the whole polycistronic messenger during chloramphenicol treatment (8).

We observed that E.coli XA 7007 (suA$^-$) did not synthesize deoxynucleotide kinase mRNA after T4 infection in the presence of chloramphenicol, as shown by the enzyme forming capacity method. Furthermore, Young (25) showed by hybridization experiments that the presence of the suA$^-$ allele failed to enhance significantly the amount or number of types of T4 RNA made in the presence of chloramphenicol. Since the suA gene was shown to code for the RNA termination factor rho (12), it could be concluded that the changed rho-factor in an E.coli strain carrying a suA mutation, could not conserve delayed early RNA during chloramphenicol treatment.

Thus it seems improbable that after T4 infection delayed early RNA is synthesized neither during leucine starvation, nor during chloramphenicol treatment of an E.coli strain carrying the suA mutation. The block of delayed early RNA synthesis in the presence of chloramphenicol could therefore reflect the need of phage protein for the formation of delayed

early RNA according to hypethesis II) above.

This hypothesis was supported by an experiment, where the amino acid analogue ethionine was used to block delayed early RNA synthesis. The addition of ethionine does not affect the incorporation of radioactive amino acids in T4 infected cells (16), but inhibits the synthesis of delayed early RNA. Cells treated with ethionine were allowed to accumulate early mRNA for 8 min, whereafter rifampin was added and the amino acid analogue was chased with an exess of methionine. The enzyme forming capacities of dCMP HMase mRNA and deoxynucleotide kinase mRNA were determined. It was found that the immediate early mRNA coding for dCMP HMase was synthesized during ethionine treatment. However, almost no deoxynucleotide kinase mRNA was formed. It was concluded that ethionine blocked the formation of a phage protein which is required for the formation of deoxynucleotide kinase mRNA.

The need of phage protein for the formation of delayed early RNA was finally confirmed by a superinfection system.

If E.coli cells were primarily infected with a deoxynucleotide kinase deficient mutant of T4, the cells seemed to synthesize a phage protein during the primary infection period, which could work in trans on a superinfecting wild type genome, and synthesize deoxynucleotide kinase mRNA in the presence of chloramphenicol.

It was found by the enzyme forming capacity method that deoxynucleotide kinase mRNA was synthesized during the chloramphenicol treatment.

Furthermore, it was found that dCTPase mRNA also needed phage protein for its formation, by using a similar superinfection system, where a T4 mutant, defective in dCTPase was used in the primary infection.

It was concluded that certain phage proteins, synthesized early during the infection period, control the formation of delayed early RNA.

A mutation in the *rho* gene of the host, relieves the need of phage protein synthesis for the formation of dCTPase mRNA (Paper III)

As shown above phage protein is needed for the formation of dCTPase mRNA and deoxynucleotide kinase mRNA. It is known from *in vitro* experiments (4) that *E.coli* RNA polymerase can transcribe both the immediate early genes and certain delayed early genes in a highly purified system in the absence of the host RNA termination factor *rho* (14), while only immediate early genes are transcribed in the presence of *rho* (4, 13).

These results could be interpreted to mean that there exists a *rho*-sensitive locus between the immediate early genes and certain delayed early genes. This locus must be neutralized by a phage protein with anti-*rho* activity to permit the polymerase to elongate the transcription into the delayed early region. Therefore the formation of some delayed early RNA could require the synthesis of a T4 protein which has anti-*rho* activity. If it really is so, a mutation in the gene coding for *rho* in the *E.coli* host cell would probably relieve the need of protein synthesis for the formation of certain delayed early messengers.

Das et al. (2) have isolated a mutant of *E.coli* which carries a mutation in the *rho* gene (12), rho_{ts-15}. The strain AD 1704 carries this mutation. The strain AD 1766 is isogenic

with AD 1704, except that it has the wild type allele of the rho-gene. If AD 1704 (rho_{ts-15}) was infected with T4 in the presence of chloramphenicol, we showed by the enzyme forming capacity method that dCTPase mRNA was synthesized during the chloramphenicol treatment. No dCTPase mRNA could, however, be detected in AD 1766 (rho^{+}) after T4 infection during chloramphenicol treatment.

The mutation in the rho gene could not permit the synthesis of deoxynucleotide kinase mRNA during chloramphenicol treatment.

It is concluded that phage protein(s) with anti-rho activity control(s) the formation of dCTPase mRNA.

The *mot* gene product is required for the formation of deoxynucleotide kinase mRNA (Paper III)

Mattson (7) isolated a T4 mutant, *ts*G1, which induced a reduced rate of synthesis of the products of genes 43, 45 and rIIB. The *ts*G1 mutation was temperature sensitive. It was found to define a new gene, which was called *mot*, modifier of transcription.

The mutant *far*P85 isolated by Johnson and Hall (5) was shown to carry a mutation in the same gene, as shown by a complementation test (17).

We showed that if *E.coli* cells were infected with either of these mutants at 37 C, the initiation of deoxynucleotide kinase synthesis was delayed. Furthermore, the final amount of deoxynucleotide kinase was only about 50% of that obtained after wild type infection.

The initiation of dCTPase synthesis at 37 C seemed not to be affected by a *mot* mutation, but the final amount of dCTPase was higher than that after wild type infection.

When *E.coli* cells were infected with *ts*G1 at its permissive temperature, 25 C, the same amount of deoxynucleotide kinase was synthesized as after wild type infection.

At 25 C *far*P85 is almost defective in the synthesis of deoxynucleotide kinase, while it synthesizes the same amount of dCTPase as wild type phage. It was suggested that the *mot* gene product is the protein needed for the formation of deoxynucleotide kinase mRNA .

To test this idea the superinfection system described in paper II was used. The cells were primarily infected with a mutant defective in the structural gene of a delayed early enzyme, so that no delayed early enzyme was formed during the primary infection period. During this period the proteins were synthesized which later after superinfection with wild type phage in the presence of chloramphenicol, were presumed to be needed for the formation of delayed early RNA. If the mot gene product is needed for the formation of deoxynucleotide kinase mRNA, it would be possible to block the formation of deoxynucleotide kinase mRNA in the superinfection system by performing the primary infection with tsG1 (mot$^-$) at its nonpermissive temperature, 37 C, instead of the mutant defective in deoxynucleotide kinase.

It was found by this system that primary infection with tsG1 at 37 C could not induce the formation of the protein needed for the formation of deoxynucleotide kinase mRNA upon superinfection with wild type phage in the presence of chloramphenicol. The formation of dCTPase mRNA was not inhibited by the mot mutation.
If a similar experiment was performed at the permissive temperature of tsG1, both deoxynucleotide kinase mRNA and dCTPase mRNA could be detected.

These data are interpreted to mean that the mot gene product probably governs the formation of deoxynucleotide kinase mRNA but not dCTPase mRNA.

From the works of Mattson (7) and Sauerbier et al. (17) it seems probable that the mot gene product also governs the transcription of genes 43, 45 and rIIB.

The regulatory function of regA in T4 (Paper IV).

When E.coli cells are infected with a T4 mutant carrying a mutation in an early gene both DNA synthesis and late mRNA synthesis is blocked (DNA⁻state). If the infection is performed with a T4 mutant carrying a mutation in gene 33 or 55 only late mRNA synthesis is blocked (maturation defective (MD) state).

After T4 infection the cells start to synthesize many early enzymes at about 2 min. Among these early enzymes are dCMP HMase, dCTPase and deoxynucleotide kinase. The synthesis of these enzymes is normally shut off at about 12 min. If late mRNA synthesis is blocked by a mutation in an early gene (DNA⁻state), the early enzymes mentioned above are synthesized until about 20 min (22). A T4 mutant in the DNA⁻state or MDstate which carries a mutation in the regA gene (SP62 or R9 (24, 6)) inactivates the shut off of the synthesis of many early enzymes (23, 6). This mutation leads to continued synthesis of dCMP HMase, dCTPase and deoxynucleotide kinase. A T4 mutant carrying only a regA mutation shuts off the synthesis of early enzymes at the normal time (23).

It seems improbable that a regA mutation affects transcription, because Karam and Bowles (6) showed by short pulses of radioactive uridine that the presence of a regA mutation did not affect the rate of RNA synthesis throughout infection.

We found that there seems to be a relationship between the rate of decay of dCTPase mRNA and the state of the regA allele.

The rate of decay of dCTPase mRNA was determined by an enzyme forming capacity method. The amino acid analogues 7-azatryptophan and 5-methyltryptophan were added at 4 min after T4 infection. We have shown that these analogues render the dCTPase reversibly inactive. It is known that the analogues do not affect the incorporation of radioactive leucine into protein in T4 infected cells (19). Rifampin was added 3 min later to stop further synthesis of dCTPase mRNA. The rate of decay of dCTPase mRNA was then determined by measuring the amount of dCTPase mRNA at different times after rifampin addition. Samples were withdrawn to test tubes containing tryptophan in excess, and incubated for 35 min. The amount of dCTPase at that time is proportional to the amount of dCTPase mRNA at the time of addition of tryptophan. It was found that a DNA^- mutant *am*N55 (gene 42^-) and wild type T4, respectively, showed a rate of dCTPase mRNA decay which corresponded to a half-life of 3 min.

If a *reg*A mutation was inserted in the T4 genome the rate of decay was decreased to correspond to a half-life larger than 25 min. The decreased rate of decay was seen both in the DNA^-state (SP62x*am*N55 (*reg*A^-, gene 42^-)) and the DNA^+state (SP62 (*reg*A^-)). Only in the DNA^-state dCTPase was overproduced, as mentioned above.

Our results are in agreement with those of Sauerbier and Hercules (16). They determined the over-all rate of decay of early mRNA after addition of a pulse of radioactive uridine to

E.coli cells infected with a *regA* mutant in the DNA$^-$state. We suggest that the *regA* function is involved in the degradation of early mRNA late in infection, thereby shutting off the synthesis of many early enzymes.

This suggestion is supported by the observation that, a *regA* mutant induced a threefold larger amount of dCTPase mRNA than wild type or a DNA$^-$ mutant, as shown by the enzyme forming capacity method. These data are consistent with those of Sauerbier and Hercules (hybridization techniques) (16) and Trimble and Maley (determination of the level of gene-specific transcripts by translation in an *in vitro* system) (20). The presence of the *regA*$^-$ allele seems to result in a conservation of newly synthesized mRNA late in infection. Furthermore, we have observed that the synthesis of dCTPase in cells infected with SP62x*am*N55 (*regA*$^-$, gene42$^-$) was more sensitive to rifampin addition at 18 min, than in cells infected with *am*N55.

The amber mutations in gene 42 in SP62x*am*N55 and *am*N55 can be phenotypically suppressed by 5-fluorouracil (3). This suppression leads to a functional gene 42 product (dCMP HMase). This in turn rescues phage DNA synthesis at mutant infection. The DNA synthesis can be followed by the incorporation of radioactive thymidine. The relative efficiency of suppression at different times after infection can thus reflect the rate of

the synthesis of dCMP HMase mRNA, which is translated to functional enzyme.
By the fluorouracil technique we found that phage DNA synthesis could be rescued as late as 18 min after infection with SP62x*am*N55, but hardly at all at 18 min after infection with *am*N55.

The increased stability of early mRNA due to the reduction of the rate of decay, after infection with a *reg*A mutant, made it possible to detect the synthesis of dCTPase mRNA and dCMP HMase mRNA by the rifampin and fluorouracial experiments described above.

Why did not a mutant carrying only a *reg*A mutation overproduce early enzymes ?
This could be explained by the presence of late mRNA synthesis in this case. According to Pollack et al. (11), initiation factor F3 is changed after T4 infection, so that late mRNA has higher affinity for translation than early mRNA. We suggest therefore that early mRNA could in this case not be translated in the presence of late mRNA.
This would lead to normal amounts of early gene products but a higher content of early mRNA, than normally, in cells infected with a *reg*A single mutant.

Finally, we tested if the regA$^+$ allele was dominant to the regA$^-$ allele by a superinfection system. The cells were primarily infected with a regA mutant, carrying an amber mutation in an early gene (gene 42$^-$) (DNA$^-$state). This led to overproduction of dCTPase. The cells were then superinfected with regA$^+$ phage to investigate if the superinfection could cause the shut off of dCTPase synthesis.. As primary infecting phage SP62xis42 (regA$^-$, imm$^-$, spackle$^-$, gene 42$^-$) was chosen. This mutant carries a spackle and imm-mutation besides the regA and gene 42 mutations to minimize exclusion of a superinfecting phage (18, 21). The dCTPase less mutant amE114 was chosen as superinfecting regA$^+$ phage, so that the superinfecting phage genome could not contribute to the dCTPase level. It was found that superinfection with amE114 could cause the shut off of dCTPase synthesis from 1 - 9 min.
If superinfection was performed with the same phage (SP62x is42) or SP62xamN55, carrying the regA$^-$ allele, dCTPase synthesis was not shut off.

It was concluded that the presence of the regA$^+$ allele in the superinfecting genome was required for the shut off of dCTPase synthesis. The results were interpreted to mean that the regA$^+$ allele is dominant to the regA$^-$ allele. Since the regA protein seemed to work in trans, it seems to be a diffusible protein.

In summary it was suggested that the regA protein is required for the shut off of the synthesis of dCMP HMase, dCTPase and deoxynucleotide kinase, by being involved in the degradation of the corresponding early messengers.

Regulation of T4 early RNA synthesis (Summary).

The regulation of the synthesis of three different types of early T4 mRNA was studied in this investigation. The messengers coding for dCMP HMase, dCTPase and deoxynucleotide kinase were chosen.

We have shown that the turn on of the synthesis of these messengers were regulated in three different ways.
The early mRNA coding for dCMP HMase could be formed even if protein synthesis was blocked prior to T4 infection. It could be concluded that probably no phage protein is needed for its formation. From *in vitro* experiments it was shown by Jayaraman (4) that gene 42 (coding for dCMP HMase) could be transcribed by the unmodified *E.coli* host polymerase.
We have furthermore shown that the synthesis of dCTPase mRNA and deoxynucleotide kinase mRNA need phage protein for their formation.
If the structural gene of the *E.coli* host RNA termination factor *rho* was mutated, the need of phage protein synthesis for the formation of dCTPase mRNA could be relieved. This would probably mean that the protein governing the formation of dCTPase mRNA has anti-*rho* activity.
The structural gene of deoxynucleotide kinase could not be transcribed by *E.coli* host RNA polymerase *in vitro*. We have shown that the *mot* gene in T4 probably codes for the protein needed for the formation of deoxynucleotide kinase mRNA .

The mot gene product could be similar to a sigma-factor, as suggested by Mattson, (7). This factor could be required for the RNA polymerase to recognize the delayed early promoter for the gene coding for deoxynucleotide kinase.

If E.coli cells are infected with T4 wild type the synthesis of dCMP HMase, dCTPase and deoxynucleotide kinase is shut off at 12 min. This shut off could be due to the alteration of the translational machinery by the change of initiation factor F3 (11), whereby late mRNA is preferentially translated late in infection. We found that the regA protein seems to be involved in the degradation of early messengers late in infection. The regA protein could for example be a nuclease or it could be a regulatory protein governing the synthesis of a nuclease.
The regA protein seems to be required for the degradation of all the three early messengers studied.
In summary it could be concluded that the transcriptional regulation of early genes is comlex. It seems possible that at least three different classes of early transcripts exist. Each class would be under separate control; i) immediate early (dCMP HMase mRNA), ii) delayed early under control of an anti-rho protein (dCTPase mRNA) and iii) delayed early under control of the mot gene product (deoxynucleotide kinase mRNA).

The regA protein seems to be required for the degradation of all these messengers.

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